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厦门大学

博　士　学　位　论　文

核酸分子探针信号放大新策略及其在生物分析中的应用

New Signal Amplification Strategies of Nucleic Acid Molecular Probes and Their Applications in Bioanalysis

崔　亮

指导教师姓名：杨朝勇　教授

专　业　名　称：化学生物学

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评　　阅　　人：＿＿＿＿＿＿

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New Signal Amplification Strategies of Nucleic Acid Molecular Probes and Their Applications in Bioanalysis

A Thesis Presented

by

Liang Cui

Supervisor: Professor Chaoyong James Yang

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摘 要

核酸分子探针是近年来发展起来的具有广泛应用价值和发展潜力的生物分析工具。核酸分子探针由核酸序列构建而成，通过碱基互补配对及其他非共价作用实现对目标物的识别，并通过光、电等信号将探针与目标物的识别报告出来。核酸分子探针已经被广泛应用于化学和生物学组分物质及许多物理参数的检测。目标物的广泛性显示了核酸分子探针的重要性。然而，由于核酸分子探针对目标物的检测是通过1:1结合比率下实现的信号转换，这种方式使核酸分子探针对目标物的检测灵敏度限制在一定范围之内。为了提高检测灵敏度，科学家们发展了各种各样的放大方法。近年来，以nicking酶、核糖核酸酶H（RNase H）等核酸酶辅助的信号放大方法以其设计简单、灵敏度高等诸多优点而受到越来越多的关注。然而，与此同时，这些方法的不足之处也逐渐暴露，如nicking酶辅助的方法通用性差、RNase H辅助的方法探针不稳定，目前的放大方法以传统的核酸分子探针为工具，背景信号高、灵敏度低、无法实现复杂体系中对目标物的直接检测等。为了解决这些问题，本文分别发展了核酸外切酶III（Exonuclease III, Exo III）、T7 核酸外切酶（T7 Exonuclease, T7 Exo）及氧化石墨烯辅助的循环酶切信号放大方法（cyclic enzymatic amplification method, CEAM）用于核酸及非核酸物质的高灵敏检测，并设计了氧化石墨烯保护RNA探针的方案，发展了质量放大探针用于荧光偏振高灵敏检测小分子的方法。这些核酸分子探针信号放大新策略，提高了目标物的检测物灵敏度，拓展了目标物的检测范围，使核酸分子探针在生物分析领域发挥越来越重要的作用。论文主要分为以下几个方面：

一、核酸外切酶III辅助的循环酶切信号放大方法用于高灵敏生物分析

设计了以Exo III为工具酶、线性分子信标（linear molecular beacons, LMBs）为探针的CEAM用于DNA及非核酸物质的高灵敏检测。Exo III以双链DNA为作用底物，沿3’平末端或凹陷末端逐个水解3’单核苷酸。LMBs由线性的DNA链组成，用于报告信号的荧光基团与淬灭基团分别位于3’末端及倒数第二个核苷酸上。与链取代探针相比，LMBs使荧光基团与淬灭基团足够靠近，从而提高了淬灭效率，降低了背景信号；与目标物的杂交不需要经过竞争-取代的过程，从而提高了杂交速率，促进了CEAM的发生。最终，该方法实现了0.12 pM DNA的检测。我们

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同时将CEAM用于非核酸物质-可卡因的高灵敏检测，并取得了良好的效果。

二、T7 核酸外切酶辅助的循环酶切放大方法及其在高灵敏microRNA检测中的应用

发展了T7 Exo辅助的CEAM并组合滚环扩增技术（rolling circle amplification, RCA）用于microRNA（miRNA）的高灵敏检测。T7 Exo底物专一性强，不会对单链DNA产生非特异性降解。我们首先设计了T7 Exo辅助的CEAM，同时，组合RCA技术，我们设计的双放大方法实现了12 fM的miRNA检测。该方法可用于比较不同细胞内miRNA的表达含量，有望用于临床诊断中疾病的检测。

三、氧化石墨烯保护的DNA探针用于复杂体系中循环酶切信号放大检测microRNA

发展了氧化石墨烯（graphene oxide, GO）保护的DNA探针用于复杂体系中CEAM方法检测miRNA。GO能够保护DNA不受核酸酶降解，结合DNase I对DNA的特异性水解性能，我们发展的GO保护的CEAM能够实现9 pM miRNA的检测，并能够在复杂生物样品中对miRNA进行直接定量。

四、氧化石墨烯保护的RNA探针用于生物分子的高灵敏分析

发展了使用GO保护RNA探针长期稳定存在的方法，并在此基础上，构建了GO保护的RNA核酸分子探针用于生物分子的高灵敏检测。本章证明了GO能够保护RNA探针在核酸酶、细胞裂解液及室温长期放置下稳定存在，从而发展了GO稳定的RNA探针用于RNase H辅助的CEAM，以及RNA核酸适体探针用于VEGF及茶碱的高灵敏检测。这些探针具有较高的灵敏度和选择性，更重要的是，GO保护的RNA探针不需要复杂的操作，为RNA探针的广泛使用提供了机会。

五、质量放大的变构调控探针用于荧光偏振检测小分子

设计了质量放大的变构调控探针用于荧光偏振检测小分子。本章中，我们发展一种质量放大的变构调控探针，该探针将蛋白质与小分子的核酸适体组成一条荧光标记的 DNA 链，并设计一条较短的互补链将其固定。只有蛋白质存在时，该探针无法与蛋白质结合。当小分子目标物出现时，小分子与蛋白质的共同作用将互补链取代，并分别与对应的核酸适体结合。通过这种方式，小分子的出现转换为蛋白质与其相对应核酸适体的结合，从而导致荧光偏振信号发生显著的改变，实现了荧光偏振技术对小分子的检测。

关键词：核酸适体；生物分析；核酸酶；核酸分子探针；循环酶切信号放大

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Abstract

In the recent years, nucleic acid molecular probes (NAMP) have become a class of significant bioanalytical tools for wide applications in the field of bioanalysis and biomedicine. As a biosensor, NAMP was built by nucleic acid sequences to recognize targets by base pairing and other non-covalent interactions and produce outputs through optical, electrical or magnetic signal changes. Today, NAMPs have found wide applications in sensing of various chemical and biological substances, as well as physical parameters. Successful analysis of more and more targets has demonstrated NAMP is an important bioanalysis tool. However, in traditional NAMP systems, each target molecule reacts with a single copy of the probe, thereby limiting the detection sensitivity. In order to improve the sensitivity, a variety of amplification strategies have been proposed. Among these, nucleases (nicking endonuclease, RNase H etc.)-assisted signal amplification strategies, in which one target molecule leads to many cycles of target-dependent nuclease digestion of reporter molecules, have attracted more and more attentions for simple, convenient and ultrasensitive nucleic acid detection. Unfortunately, while nicking enzyme is sequence-specific and RNase H requires the RNA probes, the application is limited to the number of DNA sequences that can be detected and the special and tedious manipulation is needed. Meanwhile, most of the presented signal amplification strategies are based on the conventional NAMPs, such as displacing probes and molecular beacons, resulting in a high background signal, low hybridization kinetics, poor sensitivity, limited targets and failed detection in complex biological samples. In order to solve these problems, in our work, we developed exonuclease III (Exo III), T7 exonuclease (T7 Exo) and graphene oxide (GO)-assisted cyclic enzymatic amplification method (CEAM) for high sensitive, universal detection of various targets in complex biological samples. We also proposed an effective way to design robust RNA biosensors by simply mixing RNA probes with GO for analysis of

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nucleic acids, proteins, and small molecules, developed mass amplifying probe for sensitive fluorescence anisotropy detection of small molecules. The main works are listed as follows:

1. Exonuclease III-assisted cyclic enzymatic amplification method for highly sensitive bioanalysis

In this work, we proposed exonuclease III (Exo III) and linear molecular beacons (LMBs)-assisted cyclic enzymatic amplification method (CEAM) for highly sensitive detection of DNA and non-nucleic acid targets. Exo III hydrolyzes mononucleotides from the 3'-hydroxyl terminus of duplex DNA, LMBs are linear oligonucleotide probes with a fluorophore and quencher attached to 3' terminal and penultimate nucleotides, respectively. Compared to displacing probes, for the LMBs, because both quencher and fluorophore are attached to the same strand with adjacent positions, efficient quenching can be obtained with very low probe background signal, meanwhile, LMBs allow faster reaction rates for there are no displacing process. Using LMBs in cyclic Exo III amplification assay, ultrasensitive nucleic acid detection methods were developed with a detection limit of less than 120 fM. Furthermore, LMBs can be extended as universal probes for detection of non-nucleic acid molecules such as cocaine with high sensitivity and excellent selectivity.

2. T7 Exonuclease-assisted cyclic enzymatic amplification method and the application in ultrasensitive miRNA analysis

We developed a T7 Exo-assisted CEAM, then, combining with rolling circle amplification (RCA) technique, a dual amplification method for isothermal amplified detection of miRNA with high sensitivity and selectivity was proposed. T7 Exo shows excellent specificity on dsDNA, thus, with designed T7 Exo assisted-CEAM and RCA, we developed a dual amplification for ultrasensitive miRNA analysis. By this way, a 12 fM miRNA can be detected directly. This approach, a promising miRNA quantification method in clinical diagnostics for its high sensitivity and selectivity, can be used to compare the expression level of different cells.

3. Graphene oxide-protected DNA probes for high sensitive microRNA analysis

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in complex biological samples based on a cyclic enzymatic amplification method

Based on graphene oxide (GO)-protected DNA probes, we have developed a cyclic enzymatic amplification method for sensitive miRNA detection in complex biological samples. DNA probes can absorb on graphene oxide (GO) thereby being protected from DNase I degradation. Because DNase I can only digest DNA but not active RNA, we developed a GO-protected and DNase I-assisted CEAM for high sensitive and multiplex microRNA analysis in complex biological samples. With this approach, as low as 9 pM miRNA can be detected with excellent selectivity. Meanwhile, the method can also be used for detection of miRNA directly in complex biological sample.

4. Graphene oxide-protected RNA probes for high sensitive bioanalysis

We have developed GO-stabilized RNA probes for high sensitive bioanalysis. In this chapter, we demonstrated that GO can protect RNA probes from nuclease hydrolysis, cell lysis digestion, or even long-term incubation in the lab. Based on this property of GOs, we proposed an effective way to fabricate stable RNA probes for RNase H-assisted signal amplification detection of DNA, and RNA aptasensor for detection of VEGF and theophylline. All the biosensors are sensitive and selective. More importantly, all the procedures do not require complicated manipulation. We believer our method will provide new opportunities to utilize the large number of RNA NAMPs.

5. Mass amplifying allosteric probe for sensitive fluorescence anisotropy detection of Small Molecules

Here we proposed a mass amplifying allosteric probes for small molecules fluorescence anisotropy (FA) detection. A mass amplifying probe (MAP) consisted of a targeting aptamer domain against a target molecule and molecular mass amplifying aptamer domain for the amplifier protein. The probe is initially rendered inactivate by a small blocking strand partially complementary to both target aptamer and amplifier protein aptamer so that the mass amplifying aptamer domain would not bind to the amplifier protein unless the probe has been activated by the target. As a result, the FA detection of small molecules converts to the FA detection of proteins,

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resulting in a molecular mass amplifying FA aptamer probes for small molecule analysis.

Keywords: Aptamer; Bioanalysis; Nuclease; Nucleic acid molecular probes; CEAM

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